METHODS AND ANIMAL MODEL FOR ANALYZING AGE-RELATED MACULAR DEGENERATION

This application claims priority to U.S. provisional application Serial No. 60/422,096, filed October 30, 2002, incorporated herein in its entirety.

FIELD OF THE INVENTION

The invention relates to methods of determining the pathology of age-related macular degeneration and methods of testing treatment protocols and candidate drugs for age-related macular degeneration. More particularly, the invention relates to use of Ccl2-deficient, Ccr2-deficient, or both Ccl2 and Ccr2-deficient mice to analyze the pathology and treatment of age-related macular degeneration and test candidate drugs for treatment of age-related macular edema.

BACKGROUND OF THE INVENTION

Age-related macular degeneration (AMD) is the principal cause of legal blindness in the United States and Western Europe. It affects over 11 million people in this country alone, and with the aging population will exact an even greater toll. The earliest visible abnormality in AMD is the accumulation of drusen (Gass, J. D. (1972) *Trans Am Ophthalmol Soc* 70, 409-36.), lipoproteinaceous deposits between the retinal pigment epithelium (RPE) and Bruch's membrane, the extracellular matrix between the RPE and the underlying choroid. Drusen are a significant risk factor for progression to choroidal neovascularization (CNV), the principal cause of vision loss in AMD (Macular Photocoagulation Study Group (1997) *Arch Ophthalmol* 115, 741-7). There is no animal model of drusen resembling that of patients with AMD. Drusen-like deposits in elderly primates (Hope, et al., (1992) *Br J Ophthalmol* 76, 11-6.) are dissimilar to human drusen both in ultrastructural morphology and biochemical composition (Hirata, A. & Feeney-Burns, L. (1992) *Invest Ophthalmol Vis Sci* 33, 2079-90; Mullins, R. F. & Hageman, G. S. (1997) in *Degenerative Retinal Diseases*, ed. LaVail, M. (Plenum Press, New York), pp. 1-10.). Attempts to create a murine model of drusen by high fat diet, disrupting the apolipoprotein E gene, inducing protoporphyria (Gottsch et al., (1993) *Arch Ophthalmol* 111, 126-9.), accelerating senescence (Majji, et al., (2000) *Invest Ophthalmol Vis Sci* 41, 3936-42), or combinations of the above (Dithmar et al., (2001) *Arch Ophthalmol* 119, 1643-9) have not succeeded in creating drusen.

The biogenesis of drusen involves RPE dysfunction, impaired digestion of photoreceptor outer segments, and subsequent debris accumulation (Hageman, et al.,. (2001) *Prog Retin Eye Res* 20, 705-32). The presence of complement C5, immunoglobulins, apolipoprotein E, vitronectin, and clusterin in human drusen (Loffler, et al., (1986) *Graefes Arch Clin Exp Ophthalmol* 224, 493-501; Hageman, G. S., et al., (1999) *FASEB J* 13, 477-84; Hageman, G. S. & Mullins, R. F. (1999) *Mol Vis* 5, 28;Johnson, et al., (2000) *Exp Eye Res* 70, 441-9; Mullins et al., (2000) *FASEB J* 14, 835-46; and Anderson, et al., (2001) *Am J Ophthalmol* 131, 767-81) suggests that focal concentration of these materials may produce a powerful chemotactic stimulus for leukocytes, possibly acting *via* a complement cascade (Killingsworth, et al., (2001) *Exp Eye Res* 73, 887-96). Consistent with this, macrophages appear to preferentially engulf the wide-banded collagen of basal deposits in patients with AMD, suggesting a role in drusen clearance (Loffler, K. U. & Lee, W. R. (1986) *Graefes Arch Clin Exp Ophthalmol* 224, 493-501; Killingsworth, et al., (1990) *Eye* 4, 613-21; Penfold, P. L., et al., (1985) *Graefes Arch Clin Exp Ophthalmol* 223, 69-76; and van der Schaft, et al., (1993) *Br J Ophthalmol* 77, 657-61). Laser photocoagulation induced regression of drusen in humans (Ho, et al., (1999) *Ophthalmology* 106, 1367-73; and Olk, et al., (1999) *Ophthalmology* 106, 2082-90) is believed to result from recruitment of macrophages that resorb these deposits (Duvall, J. & Tso, M. O. (1985) *Arch Ophthalmol* 103, 694-703).

The lack of a faithful animal model of AMD has hampered both the study and treatment of age-related macular degeneration. Thus, there is a need for a faithful animal model of drusen development and accumulation to provide mechanistic insights into the development of AMD and assist in evaluating candidate drugs for the treatment of age-related macular degeneration.

SUMMARY OF THE INVENTION

In one aspect of the invention there is provided a method for testing a candidate drug for treatment or prevention of age-related macular degeneration comprising administering the candidate drug to a Ccl2-deficient, Ccr2-deficient- or a Ccl2-deficient and -Ccr2-deficient mouse and analyzing the eye of the mouse for development or regression of drusen and/or lipofuscin accumulation therein, for affect of the candidate drug on Bruch's membrane and/or choroidal neovascularization of the eyes of the mouse.

There is also provided a method of screening a test compound for potential utility for treatment of age-related macular degeneration, comprising: (a) providing a mouse comprising a disrupted Ccl2 and/or CCR2 gene, wherein the mouse is homozygous for the disrupted gene or genes, and wherein the mouse exhibits drusen and/or lipofuscin deposits, retinal degeneration, and/or choroidal neovascularization in at least one eye at about nine to twenty-four months of age compared to a wild-type mouse that does not have the disrupted gene; (b) administering the test compound to the mouse; (c) determining the effect of the test compound on drusen, lipofuscin deposition, retinal degeneration, or choroidal neovascularization in at least one eye of the mouse; and (d) correlating the effect of the test compound on drusen, lipofuscin accumulation, retinal degeneration, and/or choroidal neovascularization with a potential utility to treat age-related macular degeneration.

In another aspect of the invention there is provided a method of monitoring the effects of expression of a Ccl2 gene in at least one eye of a Ccl2-/- mouse comprising (1) introducing a plurality of stem cells obtained from a wild type mouse into the Ccl2-/- mouse to obtain a transplanted mouse, wherein said stem cells express wild type Ccl2; and (2) observing at least one eye of the transplanted mouse for the effect of the wild type Ccr2 gene expression on drusen or lipofuscin deposition, retinal degeneration, or choroidal neovascularization in at least one eye of the transplanted mouse. There is also provided a method of a method of monitoring the expression of a Ccr2 gene in at least one eye of a Ccr2-/- mouse comprising (1) introducing a plurality of stem cells obtained from a wild type mouse into the Ccr2-/- mouse to obtain a transplanted mouse, wherein said stem cells express wild type Ccr2; and (2) observing at least one eye of the transplanted mouse for the effect of the wild type Ccr2 gene expression on drusen or lipofuscin deposition, retinal degeneration, or choroidal neovascularization in at least one eye of the transplanted mouse. There is also provided a method of monitoring the effects of expression of a Ccl2 gene, Ccr2 gene or both in at least one eye of a Ccl2 deficient, Ccr2 deficient mouse comprising (1) introducing a plurality of stem cells obtained from a wild type mouse into the Ccl2 deficient, Ccr2 deficient mouse to obtain a transplanted mouse, wherein said stem cells express wild type Ccl2 and Ccr2; and (2) observing at least one eye of the transplanted mouse for the effect of the wild type Ccl2 and/or Ccr2 gene expression on drusen or lipofuscin deposition, retinal degeneration, or choroidal neovascularization in at least one eye of the transplanted mouse.

In a further aspect of the invention there is provided a Ccl2-deficient/CCR2-deficient dual knockout mouse.

The present invention also provides a method of identifying mutations in the Ccl2 gene, Ccr2 gene or both comprising (1) obtaining an AMD DNA library or genomic DNA from a blood sample of an AMD patient; (2) screening the AMD DNA library or genomic DNA for sequences that hybridize under high stringency conditions to a wild type Ccl2 gene, Ccr2 gene, or both; and (3) sequencing the sequences that hybridize to determine the identity of any mutations contained therein.

In a further aspect of the invention there are provided expression vectors comprising SEQ ID NO. 9 and/or SEQ ID NO. 10.

In yet a further aspect of the invention there is provided a method of .screening for mutations that potentially cause or affect the development of AMD in a human comprising (1) obtaining an AMD DNA library or genomic DNA from a blood sample of an AMD patient; (2) screening the AMD DNA library or genomic DNA for sequences that hybridize under high stringency conditions to a wild type C5 receptor gene or C5a receptor gene; (3) sequencing the sequences that hybridize to determine the identity of any mutations contained therein.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. $Ccl2^{-/-}$ and $Ccr2^{-/-}$ mice develop early AMD. (**a**) Fundus photo of 15-month-old $Ccl2^{-/-}$ mouse. Inset shows higher magnification. (**b**) Drusen deposits in knockout mice increase with age ($n = 4$). (**c**) Collagen and elastin fibers (asterisks) of thickened Bruch membrane (indicated by bracket) in 9-month-old $Ccl2^{-/-}$ mouse are disrupted, and choriocapillaries are highly fenestrated (arrowheads). (**d**) Bruch membrane is thickened in 10- to 12-month-old knockout mice ($n = 5$). Asterisk $P < 0.05$. (**e**) TIMP-3 (red) immunoreactivity in RPE and Bruch membrane (BM) of 14-month-old $Ccl2^{-/-}$ mouse. There was no staining in photoreceptors (PR) or choroid (CH). (**f**) Lipofuscin autofluorescence (red) in light micrograph of RPE (arrow) of 15-month-old $Ccl2^{-/-}$ mouse. (**g**) Lipofuscin granules (arrows) in electron micrograph of 15-month-old $Ccl2^{-/-}$ mouse. (**h**) MALDI spectrum of RPE of 12-month-old $Ccl2^{-/-}$ mouse, showing A2E signal. NPP, *N*-perfluoroalkyl pyridine. Scale bar = 0.5 μm (**c**), 50 μm (**e**), 10 μm (**f**), or 2 μm (**g**).

Figure 2. $Ccl2^{-/-}$ and $Ccr2^{-/-}$ mice develop retinal degeneration. a, Fundus of an 18-month-old $Ccr2^{-/-}$ mouse shows geographic atrophy (arrows). b,c, Electron micrographs show healthy photoreceptor cell bodies in 14-month-old wild-type mouse (b) and attenuated photoreceptors with pyknotic nuclei (arrows) in 16-month-old $Ccl2^{-/-}$ mouse (c). d,e, Orderly arrays of photoreceptor outer segments in 14-month-old wild-type mouse (d) and marked degeneration and segments (asterisk) with pigment-laden RPE cells (arrows) amidst disorganized tissue in 16-month-old $Ccl2^{-/-}$ mouse (e). f, RPE of 16-month-old $Ccl2^{-/-}$ mouse shows marked vacuolization (black arrows), degenerated nucleus (black asterisk), and few pigment granules (white arrow). Choroid is filled with abundant melanocytes (white asterisks) but no choriocapillaris vessels. g,h, Retina in *Ccl2*$^{-/-}$ mouse outside these atrophic areas contains normal photoreceptor cell bodies (g) and outer segments (h). Scale bar 10 μm (b,c,f,g) and 5 μm (d,e,h).

Figure 3. *Ccl2*$^{-/-}$ and *Ccr2*$^{-/-}$ mice develop neovascular AMD and overexpress VEGF in RPE. **a-c,** Electron micrograph in 20-month-old *Ccl2*$^{-/-}$ mouse shows dilated choriocapillaries (CC) inserting processes (blue arrows) into Bruch's membrane (BM), with fragmented collagen and elastin layers (asterisks) of BM in a 20-month-old *Ccl2*$^{-/-}$ mouse. Inner BM (white arrowheads) is intact whereas outer BM (black arrowheads) is breached by choriocapillary processes (blue arrows) and fractures (red arrows). Higher magnification of insets (white area - **b** and black area - **c**) shows breaks (red arrows) in outer BM and endothelial processes (blue arrows) inserted into BM, disrupting outer collagenous (black asterisk) and elastin and inner collagenous layers (white asterisks), and large fenestrae (arrowheads) (**c**). **d-f,** CNV in 24-month-old *Ccr2*$^{-/-}$ mouse where an endothelial cell (E) and fibrocytes (asterisks) invade sub-RPE space through a defect in BM (arrowheads), disrupting overlying photoreceptors (PR). Higher magnification of insets shows (**e**) fibrocytes (F) invading BM and disrupting overlying RPE (r) extracellular matrix, and (**f**) an endothelial cell (E) and fibrocyte processes (asterisks) that have broken through a discontinuity in BM (arrowheads) to displace an RPE cell (R) from its intact monolayer (r). VEGF staining (blue) is minimally present in RPE of 18-month-old wild-type (**g**) but markedly expressed in RPE and choroid of 18-month-old *Ccl2*$^{-/-}$ mouse (**h**). Scale bars 2 μm (**a,e,f**), 1 μm (**b,c**), 10 μm (**d**), and 100 μm (**g,h**). **i-l,** Intrachoroidal neovascularization leaks indocyanine green but not fluorescein.

i, Late phase (12 min) fluorescein angiogram corresponding to area in **a-c**

shows no leakage (arrow) in the region whereas **j-l,** indocyanine green angiography reveals a focal area (arrow) of hyperfluorescence that increases over time (**j**-3 min, **k**-6 min, **l**-10 min). **m,n,** Choroidal neovascularization leaks fluorescein. **m,** Fluorescein angiography shows focal early (2 min) hyperfluorescence (**m**) that increases both in intensity and area in the late (9 min) frame (**n**) corresponding to region in **d-f**.

Figure 4. Complement proteins and IgG deposition in *Ccl2*$^{-/-}$ and *Ccr2*$^{-/-}$ mice, and C5a and IgG stimulate Ccl2 and VEGF secretion in RPE cells and CEC. **a,** Complement C5 (blue) staining in RPE and choroid (CH) of 18-month-old *Ccr2*$^{-/-}$ mouse. **b,** IgG staining (blue) in choroid and RPE in 14-month-old *Ccl2*$^{-/-}$ mouse. **c,** Colocalization of complement C3c (red) and IgG (green) around choroidal vessel (V) wall and in RPE of 14-month *Ccl2*$^{-/-}$ mouse. Merged picture shows yellow costaining. **d,** Vitronectin immunoreactivity in RPE and choroid of 18-month-old *Ccr2*$^{-/-}$ mouse. **e,** CD46 staining in RPE of 14-month-old *Ccl2*$^{-/-}$ mouse. **f,** Serum amyloid P component staining in RPE and choroid of 14-month *Ccl2*$^{-/-}$ mouse. RPE, asterisks. Choroid, CH. Scale bar 100 μm (**a,b**), 25 μm (**c**), 50 μm (**d-f**). **g,** Western blot. RPE and choroid lysates from 6-month-old wild-type (Young WT), 18-month-old wild-type (Old WT), 6-month-old *Ccl2*$^{-/-}$ (Young CCL2), 16-month-old *Ccl2*$^{-/-}$ (Old CCL2), 6-month-old *Ccr2*$^{-/-}$ (Young CCR2), and 18-month-old *Ccr2*$^{-/-}$ (Old CCR2) mice were analyzed by antibody against mouse IgG. A 23 kD reactive fragment corresponding to IgG light chain was identified. **h,** Ccl2 release at 24 h from C5a-stimulated RPE cells and IgG-stimulated choroidal endothelial cells (CEC). **i,** C5a and IgG upregulate RPE secretion of VEGF at 8 h. Asterisks $P < 0.05$.

Figure 5. Ccl2 overexpression and macrophage infiltration in aged wild-type mice. Ccl2 fluorescence (blue) is not observed in 4-month-old wild-type (**a**) but marked immunoreactivity is present in RPE and choroid of 12-month-old wild-type mouse (**b**). Cluster of F4/80 positive (blue) macrophages in choroid of 12-month-old wild-type (**c**) but not in 16-month-old $Ccl2^{-/-}$ mouse (**d**). Scale bar 150 μm (**a,b**) and 15 μm (**c,d**). **e,** Percentage of choroidal cells expressing F4/80 (macrophages) in young (3-month-old; white bars) and old (12-month-old; black bars) wild-type and knockout mice. $n = 4$. Asterisk $P < 0.01$. **f,** Western blot. RPE and choroid lysates from 6-month-old wild-type (Young WT), 18-month-old wild-type (Old WT), 6-month-old $Ccl2^{-/-}$ (Young CCL2), 16-month-old $Ccl2^{-/-}$ (Old CCL2), 6-month-old $Ccr2^{-/-}$ (Young CCR2), and 18-month-old $Ccr2^{-/-}$ (Old CCR2) mice were analyzed by antibody against mouse C5aR. A 50 kD reactive fragment corresponding to a reduced C5a receptor fragment was identified.

Figure 6. Macrophages are immobilized by, adhere to, and degrade C5 and IgG. **a,** Migration of wild-type peritoneal macrophages, toward Ccl2, across membranes coated with CIV and BSA, C5a, or IgG. * $P < 0.05$, # $P < 0.01$ compared with BSA. $n = 3$. **b,** Adhesion of wild-type peritoneal macrophages to slides coated with CIV and C5a or IgG. * $P < 0.05$, # $P < 0.01$ compared to BSA. $n = 3$. **c,d,** Choroidal macrophages of 12-month-old wild-type mice clear C5 and IgG *in situ*. Quantitation shows significantly less C5 (**c**) and IgG (**d**) immunoreactivity in sections from 12-14-month-old knockout mice incubated with macrophages (Mϕ) compared with sections without macrophages. * $P < 0.05$, # $P < 0.01$. $n = 4\text{-}7$. **e-g,** Confocal images from 12-month-old $Ccr2^{-/-}$ mouse eye section incubated with wild-type choroidal macrophages for 2 h. An F4/80 positive (blue) macrophage adheres to the section (**e**). IgG-immunoreactive material (red) (**f**) seems

closely associated with and engulfed by macrophage in the merged image (**g**). Scale bar 15 μm.

Figure 7A-D is the nucleotide sequence of the human Ccl2 gene (variants, promoter, and enhancer regions) (SEQ ID NO. 1-4).

Figure 8A-D is the nucleotide sequence of the human Ccr2 gene (variants, isoforms, promoter region) (SEQ ID NO. 5-8)

Figure 9 is the nucleotide sequence of the human C5 receptor gene.

Figure 10 is the nucleotide sequence of the human C5a receptor gene.

DETAILED DESCRIPTION OF THE INVENTION

The inventors have discovered two strains of genetically modified mice that develop many features of AMD as they age. Elderly mice (9 - 24 months) deficient in the gene for monocyte chemoattractant protein-1 (Ccl2, formerly referred to as MCP-1) (Lu, B et al., (1998) *J Exp Med* 187, 601-8) or its cognate receptor CC chemokine receptor-2 (Ccr2) (Kuziel, et al., (1997) *Proc Natl Acad Sci U S A* 94, 12053-8.) develop drusen, lipofuscin, and thickened Bruch's membrane (the extracellular matrix between the RPE and choroid), the earliest manifestations of AMD in humans, as well as intrachoroidal neovascularization. They also develop degeneration of the outer neural retina, which is seen in many patients with AMD (Green, W. R. & Enger, C. (1993) *Ophthalmology* 100, 1519-35). These pathologies are absent in age-matched wild-type mice and several other knockout strains of mice.

The present inventors have discovered that the development of drusen is more pronounced in the Ccl2 mice in comparison to the Ccr2 mice. Also, the accumulation of drusen occurs earlier in the Ccl2 mice. However, Ccr2 -/- mice also display evidence of drusen on fundus examination (Fig. 1). Just as Ccl2 deficient mice, Ccr2-deficient mice also exhibit phenotypic variation: some have the discrete hard drusen, while others have confluent drusen.

The subretinal deposits observed in the Ccl2 and Ccr2 mice have ophthalmoscopic and angiographic (Fig. 1) characteristics similar to drusen in AMD. Some deposits are discrete while others are confluent like hard or soft drusen, respectively, in patients with AMD (Fig. 1). The deposits are histologically similar to the human counterpart and absent in wild-type mice (Fig. 1). Bruch's membrane is visibly thickened in the knockout mice as in AMD. The choroid is markedly hypervascular and thickened, resembling the histologic appearance of intrachoroidal neovascularization (Fig. 3a-c). The outer nuclear layer of the neural retina is markedly attenuated, and photoreceptor inner & outer segments are nearly absent in many regions of the retina (Fig. 2), as seen in human AMD in regions of RPE cells compromised by drusen.

RPE cells of the knockout mice are engorged with lipofuscin (Fig. 1g), autofluorescent lysosomal storage bodies abundant in patients with AMD. Basal membranogranular deposits, the earliest pathological changes in AMD (Green et al., (1993) *Ophthalmology* 100, 1519-35; and Green, et al., (1977) *Trans Am Ophthalmol Soc* 75, 180-254) , are seen in Ccl2 -/- mice (Fig. 1). Bruch's membrane was markedly thickened and internally fragmented in these mice, with disruption of the collagen and elastin layers (Fig. 4d). The average thickness of Bruch's membrane in nine month-old knockout mice (1.8 μm) is significantly higher than in wild-type mice at the same age (0.45 μm). By comparison, in humans with AMD, the average thickness of Bruch's membrane is approximately 3 μm (Ramrattan, et al., (1994) *Invest Ophthalmol Vis Sci* 35, 2857-64). Lipofusin granules, autofluorescent lysosomal residual bodies that accumulate with age in RPE cells of human, have been implicated in AMD development (Delori et al., 2000) and are found in Ccl-2-/- mice in an age dependent fashion, as is A2E, the principal fluorophore of lipofuscin (Figure 1h).

Choroidal neovascularization (CNV) is observed in Ccl2 mice. Figure 3 shows leakage due to CNV as captured by indocyanine angiography. Figure 3a-c are transmission electron micrographs of CNV that depicts breaks in Bruch's membrane with choroidal endothelium injecting processes through these breaks. This pathology, which is identical to the earliest event in the development of CNV in human patients with AMD, has not previously been described in a spontaneous model.

Examination of human drusen revealed the presence of C5a within the deposits. It was also found that recombinant complement 5a up-regulates the secretion of Ccl2 in human RPE cells (Figure 4h). This may explain the presence of subretinal deposits in Ccl2 and Ccr2 deficient mice, which cannot recruit macrophages, which are thought to aid drusen clearance (Duvall and Tso, 1985). This provides a mechanistic link between drusen and macrophage recruitment, and suggests a causal link between the gene defects and the presence of drusen in these knockout mice.

The totality of the data suggests that macrophages play a critical role in drusen resorption, which is impaired in the absence of Ccl2 or its receptor Ccr2. The presence of both drusen and CNV (the respective key findings of both types (non-exudative and exudative) of macular degeneration) in these mice at an age similar to human (adjusted for species longevity) makes this an attractive model for investigating AMD and the role of senescence. This model not only provides evidence for a macrophage role in drusen clearance, but also provides a powerful platform to study the molecular etiology of AMD and the effect of candidate drugs or treatments on the development or progression of AMD.

Current animal models of CNV (the neovascular form of AMD that accounts for over 80% of visual loss in patients with AMD) relying on laser injury to fracture Bruch's membrane or viral transfection of VEGF into RPE cells, although useful for experimental study, are poor facsimiles of the human condition. Thus, particularly remarkable was the identification of CNV with frank evidence of angiographic leakage in 4 of 15 Ccl2–/– and 3 of 13 Ccr2–/– mice older than 18 months, and in none of 16 age-matched wild-type mice. This frequency of conversion to the neovascular stage is comparable to the rate of progression from drusen to CNV in humans with AMD1. At earlier stages (15-19 months), CNV had breached the outer, but not inner, aspect of Bruch's membrane (intrachoroidal neovascularization), showing angiographic leakage of indocyanine green but not fluorescein (Fig. 3a-c, i-l). This nascent angiogenesis later (18-27 months) completely breached Bruch's membrane, causing RPE and photoreceptor disruption due to the accumulation of subretinal fluid leakage from these immature vessels, which was visible on fluorescein angiography (Fig. 3d-f, m, n). It is shown in Figure 3 that VEGF was overexpressed in the RPE in senescent Ccl2 or Ccr2 deficient, but not age-matched wild-type, mice (Fig. 3g,h), consistent with its putative role as the angiogen driving CNV.

Recent evidence suggests that complement activation and immune complex deposition occur in eyes of humans with AMD. (Mullins, et al., FASEB J 14, 835-846 (2000); Johnson, et al., Exp Eye Res 70, 441-449 (2000); and Anderson et al., Am J

Ophthalmol 134, 411-431 (2002). The deposition of many of these proteins in aging Ccl2–/– and Ccr2–/– mice was observed in the present studies. Complement component C5 (Fig. 4a), immunoglobulin G (IgG) (Fig. 4b,c,g), the complement regulatory proteins vitronectin (Vn) and CD46 (membrane cofactor protein) (Fig. 4d,e), serum amyloid P component (SAP), a potential activator of the complement cascade (Fig. 4f), and advanced glycation endproducts (AGE) (data not shown) were present in RPE or choroid of both strains of knockout mice, but not age-matched wild-types, similar to their distribution in eyes with AMD. Colocalization of IgG and C3c in choroidal vessel walls (Fig. 4c) not only suggests the presence of immune complexes, but also reflects ongoing immune deposit formation because C3c, a split-product of surface bound C3b, is cleared within hours. The joint presence of CD46, a membrane-bound regulator that facilitates inactivation of the activated complement components C3b/C4b, and vitronectin, a fluid-phase regulator that binds to the terminal complement complex to regulate complement-mediated lysis, along with localization of complement intermediates suggests that complement activation occurs to completion. These deposits were identified in 6 of 7 Ccl2–/– and 4 of 6 Ccr2–/– mice as young as 6 months of age, predating the changes visible on fundus examination, consistent with a potential causal role. Such deposits were not identified in wild-type mice.

In other immune complex deposition disorders, it has been postulated that these proteins serve as an inflammatory nidus by inciting macrophage recruitment through Fc and complement receptor binding, triggering humoral activation and phagocytosis. Consistent with this hypothesis, it is shown herein that Ccl2 secretion by human RPE and choroidal endothelial cells (CEC) was upregulated by C5a (the activated form of C5) and

IgG, respectively (Fig. 4h). AGE also stimulates human RPE cell secretion of Ccl2 (ref. 27).

These data may explain the presence of subretinal deposits in Ccl2 and Ccr2 deficient mice which are impaired in recruiting macrophages requisite for clearance and degradation of drusen and other debris. Consistent with this hypothesis, there was an age-dependent increase in the expression of Ccl2 in the RPE (Fig. 5a,b), and in macrophage infiltration in the choroid of wild-type mice (Fig. 5c-e). Using flow cytometry, we found that aging was associated with a marked increase (15-fold) in the number of macrophages in the choroid of wild-types compared with only a modest (2-3 fold) increase in knockout mice (Fig. 5e). These data suggest that macrophage recruitment in aged wild-type mice is principally directed along the Ccl2-Ccr2 axis. Along with overexpression of C5 in the RPE and choroid of Ccl2–/– and Ccr2–/– mice, marked upregulation of the C5a receptor (C5aR) in both strains of knockout mice starting at an early age, and in wild-type mice at a later age was observed (Fig. 5f). These findings suggest that in the wild-type animal ongoing stimulation by C5a, which upregulates C5aR expression, leads to Ccl2 production and subsequent clearance of C5 and molecules tagged by this opsonin. The inability to summon sufficient numbers of or appropriately stimulated macrophages in knockout mice however, would lead to continued C5 deposition.

Both C5a and IgG stimulated human RPE cells to increase their secretion of the potent angiogenic cytokine vascular endothelial growth factor (VEGF) (Fig. 4i), which is consistent with RPE overexpression of VEGF in senescent Ccl2 or Ccr2 deficient mice (Fig. 3h). AGE also upregulates human RPE and CEC secretion of VEGF. Together these processes may underlie the development of CNV and highly fenestrated choroidal capillaries (Fig. 1c, 3c), both of which can be induced by VEGF in these mice.

Cell culture inserts were used to examine the migration of macrophages across a porous membrane coated with collagen IV (CIV, an abundant constituent of Bruch's membrane) in response to Ccl2. The migration of macrophages across this CIV-coated membrane when simultaneously coated with C5a or IgG was then tested to determine whether macrophages recruited to these protein-deposition sites by locally secreted Ccl2 are immobilized when they contact these proteins in the extracellular matrix. It was found that Ccl2-induced macrophage chemotaxis was inhibited both by C5a and IgG (Fig. 6a). Such immobilization indicates that macrophages adhere to C5a or IgG coated surfaces. Using CIV-coated multi-spot slides coated with C5a or IgG, it was shown that macrophages adhere to these proteins in a dose-dependent fashion (Fig. 6b). Collectively these data suggest that macrophages recruited by Ccl2 become immobilized when they contact C5a or IgG and associate with them in the extracellular matrix.

Because macrophages were immobilized by and adhered to C5 and IgG *in vitro*, and aging was associated with macrophage infiltration into the choroid of wild-type mice, it is possible that these cells scavenge immune complexes identified in the eyes of Ccl2–/– or Ccr2–/– mice. To test this hypothesis, macrophages were purified from aged wild-type choroids by magnetic cell sorting and plated on unfixed eye sections from Ccl2–/– or Ccr2–/– mice which were rich in C5 and IgG deposits in their RPE and choroids. Incubation with wild-type macrophages for 24 hours markedly reduced the total RPE/choroidal area occupied by C5 or IgG, compared with untreated sections (Figs. 6c,d). Within 2 hours, macrophages were spread out over the tissue and intimately associated with protein deposits (Fig. 6e-g). These results indicate that macrophages clear C5 and IgG deposits *in situ* and assign a pivotal role for macrophage deficiency in the accumulation of complement components and immunoglobulins in Ccl2–/– or Ccr2–/– mice.

The present invention provides the first animal model of AMD that recapitulates the key elements of the human condition in senescent mice lacking the macrophage chemoattractant Ccl2 or its cognate receptor Ccr2. The presence of similar pathology in two ligand/receptor strains that are defective in induced macrophage trafficking strengthens the hypothesis that macrophage dysfunction plays a role in its pathogenesis. The accumulation of several complement components, complement regulatory proteins, and IgG in these mutant mice, as in humans with AMD, suggests that impaired macrophage recruitment allows accretion of proteins associated with complement activation and immune complex deposition. Inability to summon macrophages is thus associated with senescence-associated development of features strongly reminiscent of human AMD, corroborated by several lines of evidence. In particular the present inventors have shown that Ccl2-driven macrophages are immobilized by and adhere to C5a and IgG in vitro, and that macrophages degrade these proteins in situ. Combined with the observation of a marked deficiency of macrophages in the choroids of aged knockout mice, these data suggest that impaired macrophage mobilization *in vivo* leads to non-clearing of these proteins since these cells are known to scavenge immune complexes via complement opsonization in vivo.

Since deposition of complement-related proteins and IgG precedes the development of drusen and lipofuscin, it is likely that AMD-like pathology is due, at least in part, to complement activation and immune complex deposition rather than the converse. Because RPE cells in eyes with AMD that are immunoreactive for complement-related proteins and IgG exhibit anatomic prelethal signs it has been suggested that accumulation of these proteins compromises RPE function The presence of IgG along with complement C3 and C5 intermediates is strongly suggestive of the presence of immune complexes, and is consistent with the presence of circulating retinal auto-antibodies in patients with AMD. Furthermore, patients with membranoproliferative glomerulonephritis, in which complement activation and immune complex deposition cause glomerular injury, develop drusen resembling AMD-associated drusen in ultrastructure and composition, including C5 and IgG deposition, as well as CNV. Collectively these findings support the concept that complement activation and immune complex deposition may injure the RPE in AMD. RPE injury, which may be manifested by secondary photoreceptor degradation, also can be triggered by excessive accumulation of lipofuscin. SAP and TIMP-3 also may impair drusen clearance by functioning as protease inhibitors. RPE overexpression of VEGF stimulated by complement components and IgG combined with fragmentation of Bruch's membrane provides an environment permissive for CNV.

The presence of both atrophic and neovascular pathologies in Ccl2–/– or Ccr2–/– mice at an age corresponding to human senescence makes these mice attractive models for investigating both early and late AMD. Because mouse retina does not contain a specialized macula, this model is not an exact replica of the human condition. However, the pathology in human AMD, while pronounced in the macular area, is not confined to this central region, and the findings observed in aged Ccl2–/– or Ccr2–/– mice closely resemble those of the clinical condition in anatomical appearance, biochemical composition, and functional disruption. More importantly, they define a system for molecular dissection of the determinants of AMD pathogenesis, and provide a platform to develop and validate novel therapeutic strategies and test compounds

Ccl2 -/- , Ccr2 -/- mice and dual knockout mice, Ccl2 -/- / Ccr2 -/- mice may be used to characterize the temporal development of AMD, preferably from ages of about 9 to about 24 months by ophthalmoscopy, angiography, and histopathology, for example, as compared to wild-type age-matched mice. In characterizing the development of AMD the eyes of these mice are systematically examined at various ages, such as for example, at 1, 3, 6, 9, 12, 18, and 24 months to characterize the temporal development of the retinal and subretinal pathology. For example, the eyes of the mice may be examined by:

1. Clinical Retinal Evaluation – examination & fundus photography through dilated pupil, e.g., 50 degree fundus photography to quantify yellow spots (drusen);
2. Fluorescein angiography – Staining or leakage within the eye may be identified;
3. Histology – Paraffin embedded and frozen sections of affected eyes may be studied for morphology and biochemical composition (lipid, cholesterol, lipofuscin);
4. Immunohistochemistry – Drusen (C5a, C5b-9, ApoE, vitronectin, clusterin staining for human correlation); Proliferating cell nuclear antigen (PCNA)+CD31 (proliferating choroidal endothelium); and/or
5. Electron Microscopy – Morphology and morphometry of various structures, e.g., photoreceptors, RPE, Bruch's membrane (integrity and thickness), choroidal vasculature may be examined.

In one aspect of the invention, the Ccl2, Ccr2 and/or Ccl2/Ccr2 (dual knockout) knockout mice may be used to test candidate drugs for treatment of AMD. Dual knockout mice are created by a series of genetic backcrosses using the cross-backcross-intercross scheme, which is well known in the art. Ccr2 -/- mice are mated with Ccl2 -/- mice to yield heterozygous F1 offspring. The F1 mice are intercrossed and the progeny screened by PCR, for example, for Ccr2 and Ccl2. B1 progeny, heterozygous for Ccr2 and Ccl2 are intercrossed, and mice homozygous for both disrupted genes are selected for example, by PCR typing for continued backcrossing. Mice are genotyped by any method, such as by analyzing tail DNA samples using Southern blot strategies or by PCR analysis with multiprimer sets that amplify in the disrupted gene, transgene insert or neomycin resistance gene insert.

Candidate drugs include pharmaceutical compounds, small molecules, peptides, antibodies, antibody fragments and nucleic acids, including oligonucleotides and polynucleotides in sense or antisense orientation and aptamers. In this aspect of the invention the candidate drug is administered to the mouse orally, systemically, e.g., intravenously, intraperitoneally, intravitreously (e.g., by injection or sustained delivery implant), transsclerally or topically, and preferably by topical application to at least one eye of a test group of Ccl2 mice, Ccr2 mice, dual knockout mice or all three types of mutant mice, and the eye(s) of the treated mice are periodically examined to determine the effect of the candidate drug on drusen accumulation, lipofuscin accumulation, Bruch's membrane or any other symptomatic marker of AMD. A decrease in drusen or lipofuscin accumulation or thinning of Bruch's membrane, an affect on retinal degeneration or choroidal neovascularization, for example, is an indication of the ability of the candidate drug to effectively treat AMD.

In one embodiment of the invention, the genetic defect is treated by introducing a wild-type gene Ccl2 or Ccr2 gene into the mouse. Chemotactic deficiency in Ccl2 -/- mice may be reversed by delivering a recombinant vector, such as for example an adeno-associated virus (rAAV) vector expressing the cDNA for Ccl2. Although Ccl2 can be delivered *via* an osmotic pump, rAAV vector administration is not only as effective as systemic administration, but also confines production and secretion of Ccl2, and is likely to restrict chemotactic activity to the eye. Reconstituting Ccl2 function *via* AAV transduction is also superior to systemic delivery as the former permits intra-animal inter-eye comparisons, thus providing greater statistical and biological fidelity to the hypothesis testing. Also rAAV vectors have demonstrated long-term, sustained high-level expression in the retina for two years, eliminating the need for pump replacement.

Similarly, the Ccr2 defect may be treated by administering a vector encoding wild-type Ccr2 gene to determine whether rescue of Ccr2 function prevents or causes regression of AMD in Ccr2 mice or dual knockout mice. Alternatively the Ccr2 defect may be corrected by stem cell transplantation of cells from Ccr2+/+ animals, either by adoptive transfer or following bone marrow ablation. Similarly, the Ccl2 defect may be corrected by stem cell transplantation of cells from Ccl2+/+ animals, either by adoptive transfer or following bone marrow ablation, for example.

The rAAV-vector cassette preferably includes a promoter, such as for example a chicken β-actin (CBA) promoter, which preferably is composed of an enhancer element or elements, such as a cytomegalovirus (CMV) immediate-early enhancer (381 bp) and a

CBA promoter-exon1-intron1 element (1,352 bp) upstream of a simian virus 40 early splice donor/splice-acceptor site, the Ccl2, gene, or both and a polyadenylation sequence, preferably the simian virus 40 polyadenylation sequence. The entire expression cassette containing the Ccl2 cDNA or Ccr2 cDNA is preferably flanked by AAV2 terminal repeats required for viral packaging. Viral vectors are packaged and purified as described (Raisler, B. J., Berns, K. I., Grant, M. B., Beliaev, D. & Hauswirth, W. W. (2002) *Proc Natl Acad Sci U S A* 99, 8909-14). The CBA promoter is preferably used as it supports expression well in both RPE cells and photoreceptors (Acland *et al.* (2001) *Nat Genet* 28, 92-5).

Efficacy of transduction by the rAAV-CBA-Ccl2, -Ccr2 or vector encoding both Ccl2 and Ccr2 may be confirmed by any method including any combination of the following:

1. *In vitro* expression: RPE cells harvested and cultured from eyes of wild-type and Ccl2 -/- mice may be probed by PCR amplification for the presence or absence of the wild-type Ccl2 transgene or Ccr2 transgene, respectively. Wild-type RPE cells and mutant RPE cells transfected with rAAV-CBP-Ccl2, -Ccr2 or vector encoding both Ccl2 and CCR2 may be subjected to PCR amplification , and optionally ELISA of the supernatant for expression of Ccl2, which is constitutively secreted (Elner, et al., (1997) *Exp Eye Res* 65, 781-9).
2. *In vivo* expression: The amount of ocular protein in mice expressed from the vector construct may be assayed after subretinal vector inoculation by ELISA about six weeks after injection. Approximately 10^{10} particles (2×10^8 infectious units) in a

volume of 1 μl of therapeutic vector is injected into one eye and the same volume of null vector in the fellow eye.

3. AAV-CBA-Ccl2, -Ccr2 or both Ccl2 and Ccr2 is injected into eyes of Ccl2 deficient mice , preferably about eight-week-old Ccl2 deficient , Ccr2-deficient mice, or dual knockout mice, and the temporal development of retinal and subretinal lesions is compared to fellow eyes injected with null vector over 24 months with interval measurements. In addition a vector such as AAV-CBA-Ccl2, AAV-CBA-Ccr2 or both or a single vector encoding both Ccl2 and Ccr2 may be injected into eyes of one-year-old Ccl2 deficient mice, one year old Ccr2 deficient mice or dual knockout mice, and the stabilization or regression of ocular lesions evaluated in comparison to fellow eyes.

In addition Ccl2 and Ccr2 function can be reconstituted by bone marrow transplantation from Ccl2 +/+ or Ccr2 +/+ mice.

In another aspect of the invention, there is provided a double knockout mouse which has both the Ccl2 and Ccr2 deletions. The mouse may be generated as described above, or by any method known to the skilled practitioner. The mouse is useful for determining the pathology of age-related macular degeneration and testing candidate drugs for treatment of age-related macular degeneration.

It is also contemplated that the genes, vectors and expression vectors of the invention may be used for stem cell transplantation to restore Ccr2 function. For example, stem cells obtained from a normal mouse, *i.e.*, containing a wild type Ccr2 gene, may be introduced either by adoptive transfer or following bone marrow ablation. For example, the normal stem cells may be introduced by intravenous injection into a

Ccr2-/- mouse or other animal. The eyes of the animal receiving the stem cell transplant are then observed to determine the effect of the transplantation. Alternatively, a Ccr2-/- mouse or other animal can be subjected to bone marrow irradiation to deplete stem cells. Following ablation of the endogenous stem cells, stem cells obtained from a wild type mouse are administered to the irradiated Ccr2-/- mouse, preferably by intravenous injection. The eyes of the transplanted mouse are then observed to determine the effect of the transplantation. Similar procedures can be employed to restore Ccl2 function in a Ccl2-/- mouse or other animal.

It is also contemplated that AMD can be treated or prevented in mammals, including humans, by administering to a patient in need, a wild type Ccr2 gene, wild type Ccl2 gene or both to compensate for a defective Ccr2 gene or Ccl2 gene or both. The wild type gene can be administered by any method known in the art, such as by administering the gene(s) *via* an expression vector, such as a replication defective adenovirus vector, directly into the eye, *via* an implant or *via* intravenous injection. Alternatively, the wild type gene can be introduced into the eye *via* stem cell transplantation as described above.

It is further contemplated that wild type Ccl2 and/or Ccr2 genes or small molecules that promote the function of Ccl2 and/or Ccr2 are used for the manufacture of a medicament for the treatment or prevention of AMD in a mammal.

It is further contemplated that the genes, vectors and expression vectors, including the promoter/enhancer regions of the genes for Ccl2 and/or Ccr2 may be used in identifying mutations or polymorphisms that place people at increased or decreased risk for developing AMD. The human Ccl2 gene, its promoter and enhancer (SEQ ID NO. 1-

4) and human Ccr2 gene and its promoter (SEQ ID NO. 5-8) are shown in Figures 7A-D and 8A-D, respectively. These sequences can be used to isolate the Ccr2 and/or Ccl2 gene from genomic DNA obtained from patients suspected of having or believed to be at risk of developing age-related macular degeneration. Also, the wild type Ccl2 and/or Ccr2 sequences or fragments thereof can be used directly or oligonucleotides based on these sequences can be generated and used to screen genomic or cDNA AMD libraries using any method known in the art. Generally, high stringency conditions are used in the screening process. Methods for screening genomic DNA and gene libraries and selection of stringency conditions are well known to those of skill in the art. *See, e.g.*, Maniatis et al., Molecular Cloning A Laboratory Manual. The isolated genes or gene fragments can then be sequenced to determine the presence of mutations in the isolated DNA. Once specific AMD mutations or polymorphisms are identified, these mutations can be used to screen patients for the presence of the mutation.

Applicants' studies have shown that C5 and C5a accumulate in the eyes of the Ccl2-/- and Ccr2-/- mice with aging, and that the inability of macrophages to clear these deposits leads to macular degeneration-like changes in the mice. Thus, defects in the C5 receptor and C5a receptor genes may promote macular degeneration. Therefore, an analysis of the C5 receptor gene and C5a receptor genes in AMD patients for the presence or absence of mutations or polymorphisms will confirm the role of these genes in the development of AMD. The sequence of each of the human C5 receptor and C5a receptor genes is shown in SEQ ID NO. 9 and 10, respectively. As discussed above for the Ccl2 and Ccr2 genes, the wild type C5 receptor and C5a receptor genes may be used to screen AMD libraries or genomic DNA obtained from AMD patients for the C5 receptor and C5a receptor genes therein and the genes so isolated can be characterized, by nucleotide sequencing to determine the presence or absence of mutations or polymorphisms, for example. Also, the C5 receptor and C5a receptor genes may be cloned into an appropriate expression vector or expression vector and further characterized.

EXAMPLES

Animals: Wild-type C57BL/6 mice (Jackson Laboratories), and Ccl2–/– and Ccr2–/– strains, generated as described previously (Lu, et al., J Exp Med 187, 601-608 (1998); Kuziel, et al, Proc Natl Acad Sci U S A 94, 12053-12058 (1997)) (incorporated herein by reference) and backcrossed 10 times to C57BL/6, were anesthetized by intramuscular injection of ketamine (50 mg/kg) and xylazine (10 mg/kg).

Fundus photography and angiography: Photographs and angiograms performed after intraperitoneal injection of fluorescein sodium (Akorn; 60 mg/kg) or indocyanine green (Sigma-Aldrich; 6 mg/kg) were captured with a TRC-50IA camera (Topcon) and evaluated by two masked readers.

Immunohistochemistry and electron microscopy: Frozen sections fixed in Histochoice MB (Amresco) and blocked with 5% donkey serum (Jackson Immunoresearch) were stained with rabbit anti-mouse C3c (1:1000 ; gift of J.D. Lambris, University of Pennsylvania, Philadelphia, Pennsylvania), mouse anti-mouse C5 (1:1000; gift of J.D. Lambris), rabbit anti-human CD46 (1:500; Santa Cruz Biotechnologies), goat anti-mouse MCP-1 (15 micro g/ml; R&D Systems), goat anti-human SAP (1:500; Santa Cruz), rabbit anti-mouse TIMP-3 (1:2500; gift of B.H.F. Weber, University of Wuerzburg, Wuerzburg, Germany), goat anti-mouse VEGF (15 micro g/ml; R&D Systems), rabbit polyclonal anti-AGE antibodies (1:1000, gift of A. Gugliucci, Touro University, Vallejo, California), or goat anti-human vitronectin (1:500; Santa Cruz). Bound antibodies were detected with Cy3-conjugated goat secondaries or Cy5-conjugated donkey secondaries (1:100; Jackson Immunoresearch). Alternatively sections were stained directly with FITC-conjugated goat anti-mouse IgG (1:100; BD Pharmingen), Cy5-conjugated donkey anti-mouse IgG (1:100; Jackson Immunoresearch) or Cy5-conjugated F4/80 (5 micro g/ml; Serotec). A "mouse-on-mouse" kit (Vector Laboratories) was used for C5 staining. Lipofuscin autofluorescence was detected through the Cy3 channel. Transmission electron microscopic studies were performed on uranyl acetate/lead citrate-stained ultrathin sections. Bruch's membrane thicknesses were measured 150 micro m from the optic nerve by averaging thinnest and thickest parts.

Western blotting: Equal amounts of total protein from RPE/choroid were resolved in SDS 4-20% polyacrylamide gradient gel and transferred to nitrocellulose membranes for western blotting with antibodies against mouse C5aR (gift of J.D. Lambris) or mouse IgG (Transduction Laboratories).

Flow cytometry: Single cell suspensions of RPE/choroids were incubated in Fc block (0.5 mg/ml; BD Pharmingen) for 15 min on ice, stained with Cy5-F4/80 antibody (1:30), and live cells were detected by gating on forward versus side scatter, followed by analysis of F4/80 in the fluorescence channel (FACScalibur, BD Biosciences).

Migration: Wild-type peritoneal macrophage migration (10,000 cells/well) toward 30 nM of mouse Ccl2 (R&D Systems) was assayed using 24-well transwell chambers (Corning) separated by a 5 micrometer polycarbonate filter coated with 50 micro g/ml collagen IV (CIV; Fluka), with or without overlay of human C5a (50 nM; Calbiochem), mouse IgG (50 micro g/well; Jackson Immunoresearch), or bovine serum albumin (BSA; 50 micro g/well; Sigma-Aldrich), by counting numbers of migrated cells after 3 hours incubation at 37 degrees C.

Adherence: Adherence of wild-type peritoneal macrophages (105 cells/spot) plated on multispot glass slides (Shandon) coated with 50 micro g/ml CIV overlaid with human C5a, mouse IgG, or BSA (0-8 micro g/spot). was quantitated using CyQuantGR (Molecular Probes) after incubation at 37 degrees C for 1 h.

Degradation: Frozen unfixed eye sections from knockout mice were transferred to 24-well culture plates and incubated with or without wild-type (12-month-old) choroidal macrophages (10,000 cells/well), purified via magnetic cell sorting using MicroBeads conjugated with CD11b antibody (clone M1/70.15.11.5; Miltenyi Biotec), for up to 24 h at 37 degrees C. Sections were fixed with Histochoice MB, stained for C5, IgG, or F4/80, and imaged by scanning confocal microscopy. Relative areas of C5 or IgG immunoreactivity were measured for 4-7 sections using image-analysis software (Photoshop, ver. 6.0; Adobe Systems).

Cell stimulation: Serum starved human CEC (gift of D.R. Hinton, University of Southern California, Los Angeles, California) and human RPE cells were stimulated with human C5a (50 ng/ml) or immobilized human IgG (50 micro g/well; Sigma-Aldrich) after attaining 80% confluence. Ccl2 and VEGF levels measured by ELISA (R&D Systems) at 8 and 24 h after stimulation were normalized to total protein.

MALDI-TOF mass spectrometry: RPE extracts and standards of synthetic N-retinylidene-N-retinylethanolamine (A2E; gifts of E. Rodriguez-Boulan, New York University, New York, New York and G.H. Travis, University of California, Los

Angeles, California) were dissolved in 50% methanol/50% water (Fisher Scientific), transferred to C18 PrepSep solid phase extraction columns (Fisher), and eluted with 1 ml methanol containing 0.1% trifluoroacetic acid (TFA; Fisher). N-perfluoroalkyl pyridine (NPP; gift of S. Rankin, University of Kentucky, Lexington, Kentucky; 250 ng) was added to samples as an external standard. The MALDI target was prepared by adding 0.5 micro l sample to deposited 0.5 micro l matrix (alpha-cyano-4-hydroxycinnamic acid; Sigma-Aldrich). Positive ion spectra were acquired on a Bruker Autoflex MALDI-TOF mass spectrometer (Bruker Daltonic). The A2E response (m/z 592.5) was normalized to the NPP response (m/z 576.1).

Statistics: Data are represented as the mean ± s.e.m. of at least 3 independent experiments and were compared using a two-tailed Student's t-test. The null hypothesis was rejected at $P < 0.05$.

EXAMPLE 1

Eyes of greater than 60 Ccl2–/– and Ccr2–/– mice and 40 age-matched wild-type mice ranging from 3 to 27 months were subjected to fundus examination. Of these, eyes from 25 Ccl2–/–, 21 Ccr2–/–, and 18 age-matched wild-type (<12 months: 6; 12-24 months: 7; >24 months: 5) mice were extensively examined histopathologically. Before 9 months of age, the fundi of Ccl2–/– and Ccr2–/– mice were indistinguishable from wild-type mice. Thereafter subretinal deposits with ophthalmoscopic and pathologic features of drusen in patients with AMD were observed in all mice of both knockout strains and increased in number with age as in humans (Fig. 1a, b). In contrast, no such changes were visible in wild-type mice even at 24 months of age (n = 5). Bruch's membrane (the extracellular matrix between the RPE and choroid) was markedly thickened in senescent Ccl2 or Ccr2 deficient mice compared with age-matched wild-types and that its collagen and elastin layers were severely disrupted with internal fragmentation (Fig. 1c), features observed in AMD. As in patients with AMD, intense immunostaining of tissue inhibitor of metalloproteinases (TIMP)-3, produced by the RPE and thought to contribute to thickening of Bruch's membrane, was observed in aged knockout mice (Fig. 1e). As Ccl2–/– and Ccr2–/– mice aged, increasing amounts of lipofuscin granules (autofluorescent lysosomal residual bodies which accumulate with age in RPE cells of humans and have been implicated in AMD development) were observed in swollen and vacuolated RPE cells (Fig. 1f, g) at 9 months and thereafter. Ultrastructural analysis of these RPE cells showed significant intracellular accumulation of dense bodies (Fig. 1h) including large ellipsoid and spherical structures of high electron density, presumably representing melanosomes and melanolipofuscin fusion particles, respectively, and numerous smaller structures of variable density representing lipofuscin granules. RPE extracts were tested for the presence of N-retinylidene-N-retinylethanolamine (A2E), the principal lipofuscin fluorophore by matrix-assisted laser desorption/ionization-time-of-flight (MALDI-TOF) mass spectrometry. RPE extracts from 12-month-old knockouts contained 25 pmol of A2E per eye (Fig. 1i). No A2E was detected in RPE of age-matched wild-type mice. Lipofuscin accumulation is thought to promote RPE dysfunction in AMD.

EXAMPLE 2

Retinal degeneration and choroidal neovascularization in Ccl2–/– and Ccr2–/– mice

As Ccl2–/– and Ccr2–/– mice aged, they exhibited several of the late findings seen in human AMD, including progressive outer retinal degeneration and CNV, similar to that seen in patients with late AMD. Despite evidence of RPE and choroidal pathology, differences in neural retinal morphology between knockout strains and wild-types were not observed before 16 months of age. At 16 months of age and thereafter, both knockout strains exhibited confluent areas of visible atrophy similar to "geographic atrophy" seen in advanced AMD (Fig. 2a). These areas were characterized by cell loss in the outer nuclear layer of the retina and atrophy of photoreceptor segments (Fig. 2b-e), as well as attenuation of the RPE and choriocapillaris (Fig. 2f) as in late AMD. In these regions the RPE was hypopigmented along with prominent vacuolization and degeneration of most intracellular organelles, and was devoid of basal infoldings. The choriocapillaris was nearly obliterated with few or no patent inner choroidal vessels observed in the areas corresponding to fundus atrophy. Regions outside these areas did not display such atrophy (Fig. 2g,h).

EXAMPLE 3

CCR2 rescue of the ocular abnormalities in Ccr2 deficient mice is accomplished by creating chimeric mice using bone marrow transplantation (BMT). *In vitro* AAV transduction results in loss of stem cell activity during infection, while *in vivo* transduction results in non-specific and low-level target expression (only 1 per 15,000 bone marrow cells are stem cells); neither approach will guarantee sustained expression *in vivo*. Ccr2 -/- mice are irradiated and repopulated with bone marrow stem cells from wildtype Ccr2 +/+ mice. Ccr2 -/- mice are maintained on antibiotic-containing water for one week before irradiation. These mice are irradiated with 900 cGy from a cesium source (delivered in two equal doses of 450 cGy 3-4 hours apart), and donor bone marrow cells (1×10^7) are injected into a tail vein. Mice are maintained on antibiotic-containing water for four weeks after transplantation. Engraftment is verified by PCR detection of the Ccr2 gene in the bone marrow of all irradiated mice. Eyes of eight-week-old chimeric mice are compared to ungrafted Ccr2 -/- mice over 24 months with interval measurements. In addition, eyes of Ccr2 -/- mice repopulated with bone marrow at one year of age are compared to ungrafted mice over the following year.

EXAMPLE 4

A candidate drug for the treatment of AMD is applied to one or both eyes of a Ccl2 mouse, which was previously confirmed to have developed AMD symptoms, e.g., drusen and/or lipofuscin deposits in the eye, thickening of Bruch's membrane. Treatment is repeated at least once daily for one to several weeks. Examination of the treated eye(s) by visual and/or fundus examination through dilated pupil is carried out periodically during treatment and the effect of treatment is compared to placebo treated wild-type eyes.